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(54) Title: IMPROVED PRODUCTION OF POLY-BETA-HYDROXYBUTYRATE IN TRANSFORMED ESCHERICHIA COLI

(57) Abstract

Methods are provided for enhancing the production of PHB from a transformed E. coli host which includes the genes coding for the PHB biosynthetic pathway. By inserting the genes coding for PHB into a host which includes a lactose utilization system, a low cost minimal media including whey can be used as the fuel and carbon source for PHB production. A plasmid, p4A, which codes for the PHB biosynthetic pathway plus four hundred extra bases on either side of the first and last genes in the pathway has been inserted into the host and has been shown to produce a larger amount of PHB accumulation in a shorter period of time than other plasmid constructs. $CaCl_2$ has been shown to be an effective agglomerating agent for agglomerating PHB which has been produced in a transformed E. coli host.

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DESCRIPTION

IMPROVED PRODUCTION OF POLY-BETA-HYDROXYBUTYRATE IN TRANSFORMED ESCHERICHIA COLI

TECHNICAL FIELD

The present invention is generally related to the production of poly-beta-hydroxybutyrate (PHB) using Escherichia coli (E. coli) which has been genetically transformed by a vector carrying the genes coding for the PHB biosynthetic pathway and, more particularly, to the more efficient production of PHB in transformed E. coli.

BACKGROUND ART

PHB is an energy storage material produced by a variety of bacteria in response to environmental stress and is a homopolymer of D-(-)-3-hydroxybutyrate which has properties comparable to polypropylene. Because PHB is biodegradable, there is considerable interest in using PHB for packaging purposes as opposed to other plastic materials in order to reduce the environmental impact of human garbage. PHB also has utility in antibiotics, drug delivery, medical suture and bone replacement applications. PHB is commercially produced from Alcaligenes eutrophus (A. eutrophus) and sold under the tradename Biopol.

As described in the article by Slater et al., "Cloning and Expression in Escherichia coli of the Alcaligenes eutrophus H16 Poly-B-Hydroxybutyrate Biosynthetic Pathway", Journal of Bacteriology, Vol. 170, No. 10, Oct. 1988, p.4431-4436, it was shown that E. coli could be genetically transformed with genes from A. eutrophus which code for the PHB biosynthetic pathway. E. coli are a far better vehicle for producing PHB than A. eutrophus since more is known about handling the bacteria, E. coli, i.e., E. coli is more easily controlled and manipulated. The transfromed E. coli were able to express PHB in relatively large quantities.

Despite PHB's advantages over other materials, its high cost of production has hindered its performance in the market. Currently, PHB is produced in transformed E. coli by growing the E. coli on luria broth (LB) and using glucose as the carbon source. Approximately one third of the production cost of PHB is attributable to the cost of the rich LB medium and the glucose. If a less expensive carbon source could by utilized, the overall cost of PHB production could be significantly reduced. In addition, much of the total cost of PHB production is attributable to purifying the PHB produced in the E. coli. Currently, PHB is purified by centrifugation, followed by mechanical lysis of the cells to release PHB, a high temperature procedure to agglomerate the PHB, and finally a spray drying step to procure the purified granules. If a less expensive method were available for collecting the PHB from the E. coli, the overall cost of PHB production could be significantly reduced.

DISCLOSURE OF INVENTION

It is therefore an object to the present invention to provide improved techniques for producing PHB in transfromed E. coli.

It is another object of this invention to provide a transformed E. coli strain which can accumulate PHB at higher levels than

previous E. coli strains and which can utilize minimal media containing whey for growing conditions.

It is yet another object of this invention to provide a method of agglomerating PHB granules from lysed E. coli cells using an ionic solution.

According to the invention, a strain of E. coli, i.e., E. coli HMS174, has been transformed by a vector containing a plasmid with the PHB biosynthetic pathway and approximately four hundred extra bases on both the upstream and downstream sides of the pathway. The HMS174 strain of E. coli was chosen because it contains a lactose utilization system and is recombination deficient so that a plasmid containing lactose genetic regions will not recombine and make the construct unstable. The lactose utilization system present in E. coli HMS174 has allowed whey to be used as a carbon source for the production of PHB. Whey is a waste product from cheese processing and is very inexpensive. Experiments have been performed which show that the strain of transformed E. coli grows in minimal media containing whey and has an average yield of PHB of approximately 85% (PHB dry weight/total cell dry weight).

In addition, experiments have been conducted which show that PHB produced in transformed E. coli may be agglomerated with various ionic solutions. To retrieve purified PHB in large quantities, the transformed E. coli cells are first lysed by mechanical or physical means, such as by sonication, or by genetic means. Then the cells are incubated in an ionic solution, such as 10 millimolar (mM) calcium chloride ($CaCl_2$), which agglomerates the PHB granules. Finally, the agglomerates are centrifuged from the culture at low speed. Experiments show that nearly all (100%) of the PHB in the culture is agglomerated and recovered by this process. The results are especially exciting since the same type of agglomeration is not possible for retrieving PHB from A. eutrophus.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects and advantages will be better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, in which:

Figure 1 is a line graph showing PHB accumulation versus time for a variety of E. coli clones containing different plasmid constructs;

Figures 2a and 2b are bar graphs showing the accumulation of PHB produced by transformed E. coli using minimal media and whey;

Figure 3 is a bar graph showing the percentage of PHB agglomeration using $CaCl_2$;

Figure 4 is a line graph showing the PHB agglomerations versus time where PHB is accumulated in the presence of radiolabelled glucose and then subjected to the agglomeration procedure; and

Figure 5 is a bar graph showing the contrasting effects of glass milk and calcium on PHB agglomeration.

BEST MODE OF CARRYING OUT INVENTION

Referring now to the drawings, and more particularly to Figure 1, it is shown that the E. coli strain HMS174 containing the plasmid p4a accumulates a greater percentage of PHB in a shorter period of time than other E. coli clones containing different plasmid constructs. The E. coli strain HMS174 is available from the Yale E. coli Stock Center, Barbara Bachman, curator. The p4a plasmid carries the PHB biosynthetic pathway and approximately four hundred extra bases to the upstream and downstream sides of the PHB biosynthetic pathway on the vector pTZ-18U. The vector pTZ-18U is available from United States Biochemicals. MSA carries the PHB biosynthetic pathway on the vector pTZ-18U and the E-lysis

gene from phage phi X 174 on another compatible plasmid. MSA differs from p4a in that it has approximately four hundred extra bases on the upstream side of the PHB biosynthetic pathway (i.e., the PHB biosynthetic pathway is cloned into pTZ-18U to create pTZ-18U-PHB called "MSA", and p4A is pTZ-18U-PHB less four hundred bases on the upstream side of the PHB biosynthetic pathway on the vector pGEM-7F+ which is available from the Promega Corporation.

The P4A, pTZ-18U-PHB (MSA), and pGEM7f-PHB(GEM) clones were all constructed from the E. coli clone harboring the PHB biosynthetic pathway discussed in the above-referenced and incorporated co-pending patent application and journal article using conventional molecular cloning techniques. As was disclosed in the patent application and journal artricle, the PHB biosynthetic pathway can be isolated from A. eutrophus and expressed in E. coli. The biosynthetic pathway is approxmately five kilobases in length and containing bases coding for beta-ketothiolase, NADP-linked acetoacetyl-coenzyme A (CoA) reductase, and PHB synthetase. Figure 1 shows that the MSA and GEM clones do not produce as much PHB as the p4A clone.

E. coli HMS174 was chosen as the host because it contains a lactose utilization system and it is recombination deficient. Recombination deficiency assures that a plasmid containing lactose genetic regions will not recombine and make the construct unstable. As will be described below, the presence of the lactose utilization system in HMS174 allows whey, a cheese manufacturing waste product whose major component is lactose, to be used as the carbon source for PHB production. In making the transformed E. coli strain, the plasmid p4a, which is the PHB boisynthetic pathway plus four hundred bases upstream and downsteam of the PHB biosynthetic pathway cloned into the United States Biochemical vector pTZ-18U, is electroplated into the E. coli HMS174. A strain of the E. coli harboring the p4A plasmid has been deposited with the American

Type Culture Cllection of 12301 Parklawn Drive, Rockville, Md. on May 23, 1990, and bears deposit number: 68329.

Experiments were performed which showed that the HMS174 strain of E. coli which had been transformed with the p4a plasmid could be grown on minimal media containing whey. The minimal media used was M9 minimal media which is described in most microbiological biology texts. Table 1 lists the formulation for a 5X concentrate of M9 minimal media where each of the listed components is added to a liter flask and water is added to one liter.

TABLE 1

5X M9 MINIMAL MEDIUM FORMULATION

30 g Na_2HPO_4

15 g KH_2PO_4

5 g NH_4CL

2.5 g NaCl

Whey was purchased from Sigma chemicals as a powder of bovine whey, and was make by stirring 20 grams of whey in water that had a final volume of 100 ml. Stirring took place with mild heating for approximately 30 minutes. This solution was then autoclaved and particulates that precipitated during centrifugation were pelleted by centrifuagtion at 10,000 x g for 10 min. The remaining supernate was used as the whey carbon source.

In the experiments, the HMS174 E. coli strain containing the plasmid p4a was inoculated from a plate culture into 50 ml of M9 minimal media + whey solution. Table 2 lists the formulation from 50 ml of minimal media containing whey at a final concentration of 8%.

TABLE 2

MINIMAL MEDIA + WHEY FOR PHB PRODUCTION

10 ml 5X M9 media
20 ml ddH_2O (double distilled water)
50 ul 1 M $MgSO_4$
5 ul 0.5% Thiamine
250 ul 20% casamino acids
20 ml 20% whey solution

The inoculated culture was grown at 37°C for 48 hours in an orbital incubator shaker at 300 rpm in a 250 ml baffled flask. After the 48 hour incubation time, the culture was stopped and the cells were harvested. Gas chromatography was used to analyze the PHB content.

Figures 2a and 2b respectively show the percentage of PHB accumulated in the cells, expressed as PHB weight per cell divided by the total weight of the cell, and the yield of PHB in the cells, expressed as the total PHB made in mg/ml, for differing concentrations of whey in solution with the minimal media. Figures 2a and 2b show that even with very low concentrations of whey, i.e., 2% in solution, high concentration of PHB accumulation (i.e., greater than 90%) and high yields of PHB (i.e., approximately 10 mg/ml). While Figures 2a and 2b show that media with higher concentrations of whey tended to produce greater concentrations and yields of PHB, it was noted that after the whey concentration exceeds 8%, PHB production begins to fall.

In the above experiments, PHB production was analyzed after forty eight hours of incubation; however, it should be noted that significant PHB production was observed after twenty four hours of incubation. In addition, it is anticipated that the relative concentrations of the Na_2HPO_4, NH_4CL, and NaCl in the 5X minimal media formulation and the relative concentrations of the 5X minimal media, double distilled water, $MgSO_4$, Thiamine, casamino acids, and

whey solution could be varied while still allowing production of PHB in a transformed E. coli host having a lactose utilization system.

Utilizing whey as the carbon source for the production of PHB, where the whey is present in minimal media, is expected to result in considerable cost savings over the prior art practice of using rich media with glucose for producing PHB. The prior art transformed E. coli cells which had plasmids coding for the PHB biosynthetic pathway, which were discussed in the co-pending patent application and journal article, could not be grown using whey as the carbon source since those bacteria did not have a lactose utilization system present therein. PHB cannot be produced using whey as its carbon source in the native host, alcaligenes eutrophus, because that bacteria also lacks a lactose utilization system. In addition as shown in Figure 1, transforming a particular E. coli host, HMS174, with a particular plasmid, p4a, allows the production of PHB at much higher percentages than when the E. coli is transformed with a different vector which also codes for the PHB biosynthetic pathway.

Because the PHB is being produced in E. coli, rather than its native host (A. eutrophus), the applicant believed that the PHB polymer produced by the transformed E. coli might have different physical properties from PHB produced in A. eutrophus. In particular, the applicant conducted experiments to determine if PHB produced by a transformed E. coli could be agglomerated by various ionic solutions. In the experiments, PHB was produced in transformed E. coli as discussed in the above-incorporated co-pending patent application and journal article. Briefly, a PHB-producing strain is grown in Luria broth (LB) containing 1% glucose for 24 hours at 37°C in a shake flask culture. The cells are pelleted by centrifugation (2,000 x g for 5 min) and then resuspended in a volume of water equal to the original culture. The cells were then lysed by sonication and various ionic reagents were added to the solution. Table 3 shows the aggregative effect on PHB produced in

transformed E. coli by various ionic solutions.

TABLE 3

AGGREGATION OF PHB BY VARIOUS IONIC SOLUTIONS

Solution*	Degree of Aggregation**
KH_2PO_4	++
NaCl	+
CsCl	-
$MgSO_4$	+++
K_2HPO_4	+
$MgCl_2$	++++
$(NH_4)_2HPO_4$	+
MgOAc	++++
NaOAc	++
KCL	-
KOAc	-
$CaCl_2$	++++
$(NH_4)OAc$	-

*All solutions were at a final conentration of 1M. **Agglomeration was subjectively graded using micrographs of each aggregate. "++++" signifies the best agglomeration and "+" signifies the lowest amount of agglomeration. "-" signifies no agglomeration.

Table 3 shows that several ionic solutions cause PHB produced in transformed E. coli to agglomerate. The best agglomerating agent was $CaCl_2$ based on a subjective judgment concerning the speed and size of the agglomerates. The agglomeration effect of $CaCl_2$ does not cause PHB produced in its native A. eutrophus to agglomerate (i.e., an experiment was performed where PHB granules were obtained from lysed Alcaligenes H16 eutrophus and subjected to calcuim chloride wherein no agglomeration was observed).

Experiments were conducted to determine the ideal concentration of $CaCl_2$ to use for agglomerating PHB. In the

experiments, the transformed E. coli cells were prepared and lysed as described above, then the solutions were brought to different mM $CaCl_2$ concentrations using a 1 M stock $CaCl_2$ solution. With low concentrations of $CaCl_2$, e.g., 1 mM, very long incubation times were required for PHB granules to agglomerate and only small agglomerates were produced. With high concentrations of $CaCl_2$, e.g., 100 mM and above, agglomeration occurred almost instantaneously and resulted in large "snowflake"-like particles that fell to the bottom of the tube. However, the agglomerates achieved with high concentrations of $CaCl_2$ appeared to have large amounts of cell debris. Therefore, high concentrations of $CaCl_2$ are not desirable for agglomeration. When meduim concentrations of $CaCl_2$ were used, e.g., 5 mM to 30 mM, agglomeration of meduim sized pellets occurred within a short incubation period of 5 to 15 minutes. Use of 10 mM $CaCl_2$ was determined to produce the best agglomeration results in terms of speed and size of agglomerate formation.

Experiments were performed to determine the percentage of PHB agglomerated by $CaCl_2$ versus the percentage of PHB left in solution. In the experiments, a PHB-producing strain of E. coli was grown in Luria broth containing 1% radio labelled glucose for 24 hours at 37°C in a shake flask culture. The cells were pelleted by centrifugation (2,000 x g for 5 min) and then resuspended in a volume of water equal to the original culture. The cells were then lysed by sonication and then the solution was brought to 10 mM by the addition of a 1 M calcium chloride stock. The tube was incubated 10 min at room temperature and then centrifuged at 400 x g for 2 min. The agglomerated PHB granules pelleted, while much of the cell debris stayed in the supernate. The supernatant was then aspirated. To determine the distribution of PHB in the pellet and supernatant, the pellet and supernatant were measured using either capillary gas chromatography or liquid scintillation counting.

Figure 3 shows that nearly all (100%) of the PHB in the culture was agglomerated and recovered by the above process. In this experiment, the amount of PHB was measured only by gas capillary chromatography. This experiment was done at several cell volumes to determine if the volume of the flask influenced the degree of agglomeration and it was found that in all volumes nearly all of the PHB was agglomerated and subsequently pelleted by centrifugation.

Figure 4 shows that it is extremely important that the culture be allowed sufficient time for agglomeration to occur, otherwise the yield is reduced. Rather than allowing a full ten minute incubation time after the solution was adjusted to 10 mM $CaCl_2$, the pellet and supernatant fractions were counted at two minute timed intervals after the adjustment. Figure 4 shows that during the first few minutes after adding $CaCl_2$ the amount of PHB present in the supernatant is actually greater that in the pellet. However, after eight minutes (where the amount of PHB measured in the pellet begins to level off), the amount of PHB in the pellet is far greater that in the supernatant fraction. It should be noted at this point that this experiment measures radioactive ^{14}Carbon, most of which is incorporated into PHB as ^{14}C-glucose (approximately 60% is incorporated), but some of which is present as soluble material. Therefore, even though nearly all of the PHB is precipitated, there is still a large number of counts in the supernate that is due to the soluble radioactive glucose.

Figure 5 shows that agglomeration of PHB can be enhanced by the addition of nucleating agents such as glass milk available from Bio 101. In Figure 5, the counts per minute (CPM) of the pellet and supernatant fractions are displayed where "+gm, +Ca" indicates PHB agglomeration in the presence of glass milk and 10 mM $CaCl_2$, "-gm" indicates PHB agglomeration in the presence of 10 mM $CaCl_2$ without glass milk, "-gm, -Ca" indicates PHB agglomeration in the absence of glass milk and $CaCl_2$, and "-Ca" indicates PHB

agglomeration in the presence of glass milk and in the absence of $CaCl_2$. From Figure 5, it can be seen that the enhancement of agglomeration by the addition of nucleating agents is not very large; therefore, larger production schemes may not be greatly benefitted by the use of such agents.

While the invention has been described in terms of its preferred embodiments where a strain of transformed E. coli has been treated which can accumulate larger quantities of PHB while using an inexpensive carbon source such as whey for PHB production and an ionic solution such as $CaCl_2$ can be used to agglomerate PHB, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims.

CLAIMS

Having thus described my invention, what I claim as new and desire to secure by Letters Patent is as follows:

1. An Escherichia coli bacterial host having a lactose utilization system transformed by a vector containing a deoxyribonucleic acid sequence coding for a poly-beta-hydroxybutyrate biosynthetic pathway.

2. An Escherichia coli bacterial host as recited in claim 1 wherein said deoxyribonucleic acid sequence includes approximately a first four hundred nucleoside bases positioned in said deoxyribonucleic acid sequence before a first of three gene sequences in said poly-beta-hydroxybutyrate biosynthetic pathway and approximately a second four hundred nucleoside bases positioned in said deoxyribonucleic acid sequence after a third of said three gene sequences in said poly-beta hydroxybutyrate biosynthetic pathway.

3. An Escherichia coli bacterial host as recited in claim 2 bearing the following ATCC deposit number: 68329.

4. An Escherichia coli bacterial host as recited in claim 1 wherein said host is derived from the Escherichia coli strain HMS174.

5. An Escherichia coli bacterial host as recited in claim 1 wherein said vector is the plasmid pTZ18U.

6. An Escherichia coli bacterial host transformed by a vector containing a deoxyribonucleic acid sequence coding for a poly-beta-hydroxybutyrate biosynthetic pathway, said Escherichia coli bacterial host being capable of using whey as a carbon source for producing poly-beta-hydroxybutyrate in recoverable quantities.

7. An Escherichia coli bacterial host as recited in claim 6 wherein said deoxyribonucleic acid sequence includes approximately a first four hundred nucleoside bases positioned in said deoxyribonucleic acid sequence before a first of three gene sequences in said poly-beta-hydroxybutyrate biosynthetic pathway and approximately a second four hundred nucleoside bases positioned in said deoxyribonucleic acid sequence after a third of said three gene sequences in said poly-beta-hydroxybutyrate biosynthetic pathway.

8. An Escherichia coli bacterial host transformed by a vector containing a deoxyribonucleic acid sequence coding for a poly-beta-hydroxybutyrate biosynthetic pathway, said Escherichia coli bacterial host being capable of using minimal media as a fuel source for producing poly-beta-hydroxybutyrate in recoverable quantities.

9. An Escherichia coli bacterial host as recited in claim 8 wherein said deoxyribonucleic acid sequence includes approximately a first four hundred nucleoside bases positioned in said deoxyribonucleic acid sequence before a first of three gene sequences in said poly-beta-hydroxybutyrate biosynthetic pathway and approximately a second four hundred nucleoside bases positioned in said deoxyribonucleic acid sequence after a third of said three gene sequences in said poly-beta-hydroxybutyrate biosynthetic pathway.

10. A medium for producing poly-beta-hydroxybutyrate in a transformed Escherichia coli host comprising minimal media and

whey, said transformed Escherichia coli host including a poly-beta-hydroxybutyrate biosynthetic pathway and capable of using said whey as a carbon source for production of poly-beta-hydroxybutyrate.

11. A medium as recited in claim 10 wherein said minimal media is aproximately twenty percent of said medium, said whey is approximately forty percent of said medium, and water is approximately forty percent of said medium.

12. A medium for producing poly-beta-hydroxybutyrate in a transformed Escherichia coli host, comprising approximately:

0.6 % percent Na_2HPO_4;
0.3 % percent KH_2PO_4;
0.1 % percent ammonium chloride;
0.05% percent sodium chloride;
58.84% percent water;
0.012% percent magnesium sulfate;
0.0005% percent thiamine;
0.01% percent casamino acids; and
40% percent whey solution.

13. A method for producing poly-beta-hydroxybutyrate, comprising the steps of:

providing a culture of Escherichia coli bacterial hosts, each host having a lactose utilization system, each host having been transformed by a vector containing a deoxyribonucleic acid sequence coding for the poly-beta-hydroxybutyrate biosynthetic pathway;

growing said culture of Escherichia coli bacterial hosts in minimal media containing whey for a periods greater than twenty four hours, each of said Escherichia coli bacterial hosts producing intra-cellular poly-beta-hydroxybutyrate;

lysing said Escherichia coli bacterial hosts in said culture

to release said poly-beta-hydroxybutyrate into solution; and

collecting said poly-beta-hydroxybutyrate.

14. A method as recited in claim 13 wherein said step of collecting includes the step of exposing said solution containing lysed Escherichia coli bacterial hosts and poly-beta-hydroxybutrate to an ionic reagent selected from the group consisting of magnesium sulfate, magnesium chloride, magnesium acetate, and calcium chloride, said ionic reagent being of sufficient concentration to agglomerate said poly-beta-hydroxybutyrate.

15. A method as recited in claim 14 wherein said ionic solution is calcium chloride at a concentration ranging between one molar and one millimolar.

16. A method as recited in claim 15 wherein said calcium chloride has a concentration of approximately ten millomolar.

17. A method for producing poly-beta-hydroxybutyrate, comprising the steps of:

providing a culture of Escherichia coli bacterial hosts, each host having been transformed by a vector containing a deoxyribonucleic acid sequence coding for the poly-beta-hydroxybutyrate biosynthetic pathway, each host being capable of using whey as a carbon source for the production of poly-beta-hydroxybutyate;

growing said culture of Escherichia coli bacterial hosts in minimal media containing whey for a period greater than twenty four hours, each of said Escherichia coli bacterial hosts producing intra-cellular poly-beta-hydroxybutyrate;

lysing said Escherichia coli bacterial hosts in said culture to release said poly-beta-hydroxybutyrate into solution; and

collecting said poly-beta-hydroxybutyrate.

18. A method for recovering poly-beta-hydroxybutyrate which has been intra-cellularly produced in a culture of transformed Escherichia coli bacterial hosts where said hosts have been transformed by a vector containing a deoxyribonucleic acid sequence coding for the poly-beta-hydroxybutyrate biosynthetic pathway, comprising the steps of:

lysing said Escherichia coli bacterial hosts to release said poly-beta-hydroxybutyrate into solution;

adding a sufficient quantity of an ionic reagent selected from the group consisting of magnesium sulfate, magnesium chloride, magnesium acetate and calcuim chloride, said sufficient quantity of said ionic reagent agglomerating said poly-beta-hydroxybutyrate in said solution; and

centrifuging said solution to pelletize said agglomerated poly-beta-hydroxybutyrate.

19. A method as recited in claim 18 wherein said ionic reagent is calcium chloride.

20. A method as recited in claim 18 wherein said calcuim chloride is present at a concentration ranging between one molar and one millimolar.

21. A method as recited in claim 20 wherein said calcuim chloride is present at a concentration of approximately ten millimolar.

22. A purified and isolated DNA sequence comprising approximately a first four hundred nucleoside bases positioned in the DNA sequence before a first of three gene sequences in a poly-beta-hydroxybutyrate biosynthetic pathway and approximately a second

four hundred nucleoside bases positioned in the DNA sequence after a third of the three gene sequences in the poly-beta-hydroxybutyrate biosynthetic pathway.

23. A plasmid designated as p4A and deposited with the American Type Culture Collection in Escherichia coli strain HMS 174 under accession number 68329.

24. The method of claim 13, wherein the vector comprises a p4A plasmid.

25. The method of claim 17, wherein the vector comprises a p4A plasmid.

26. The method of claim 18, wherein the vector comprises a p4A plasmid.

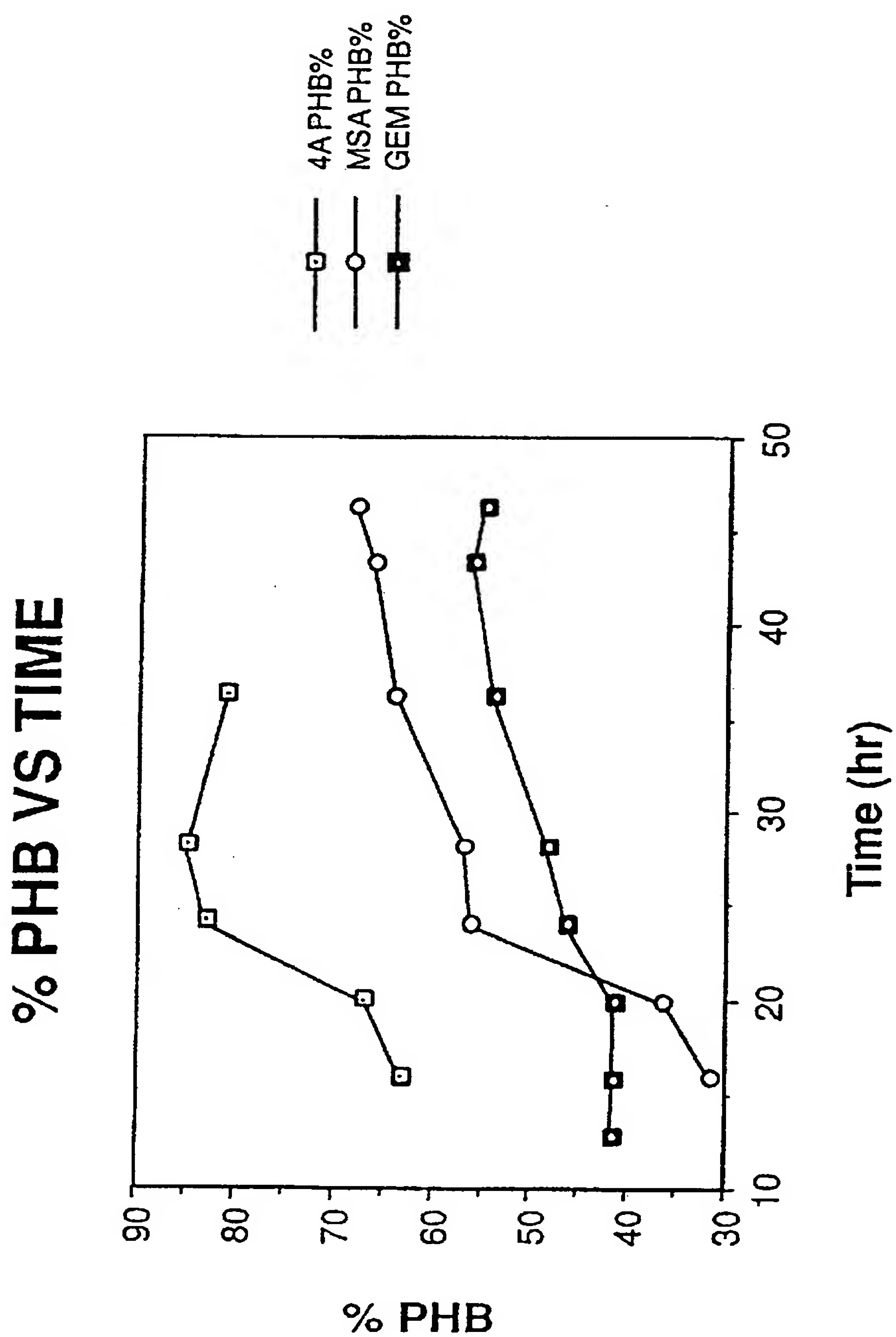
% PHB VS TIME
4A PHB%
MSA PHB%
GEM PHB%
% PHB
90
80
70
60
50
40
30
10
20
30
40
50
Time (hr)

FIG. 1

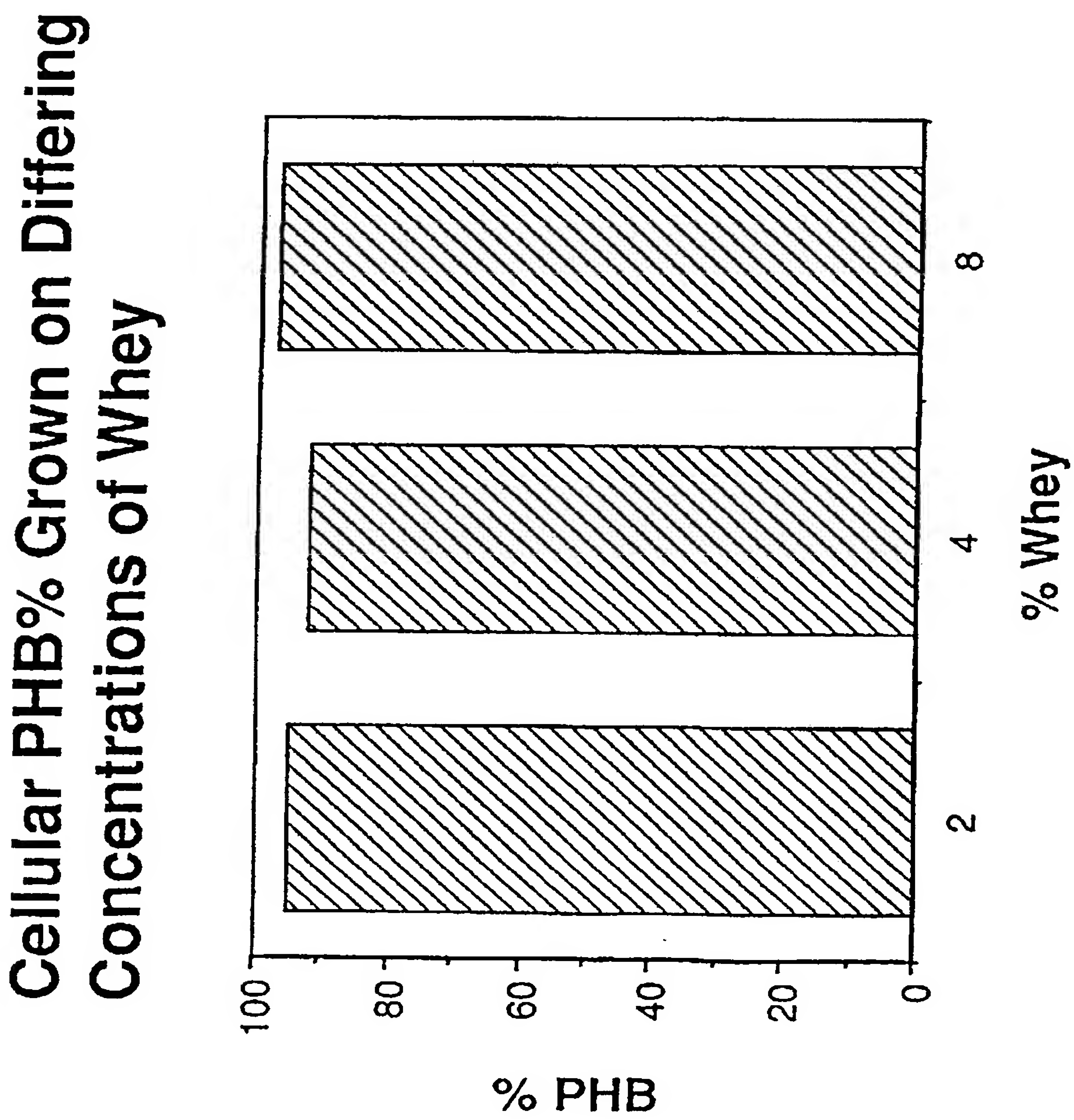
Cellular PHB% Grown on Differing Concentrations of Whey
% PHB
100
80
60
40
20
0
2
4
8
% Whey

FIG. 2a

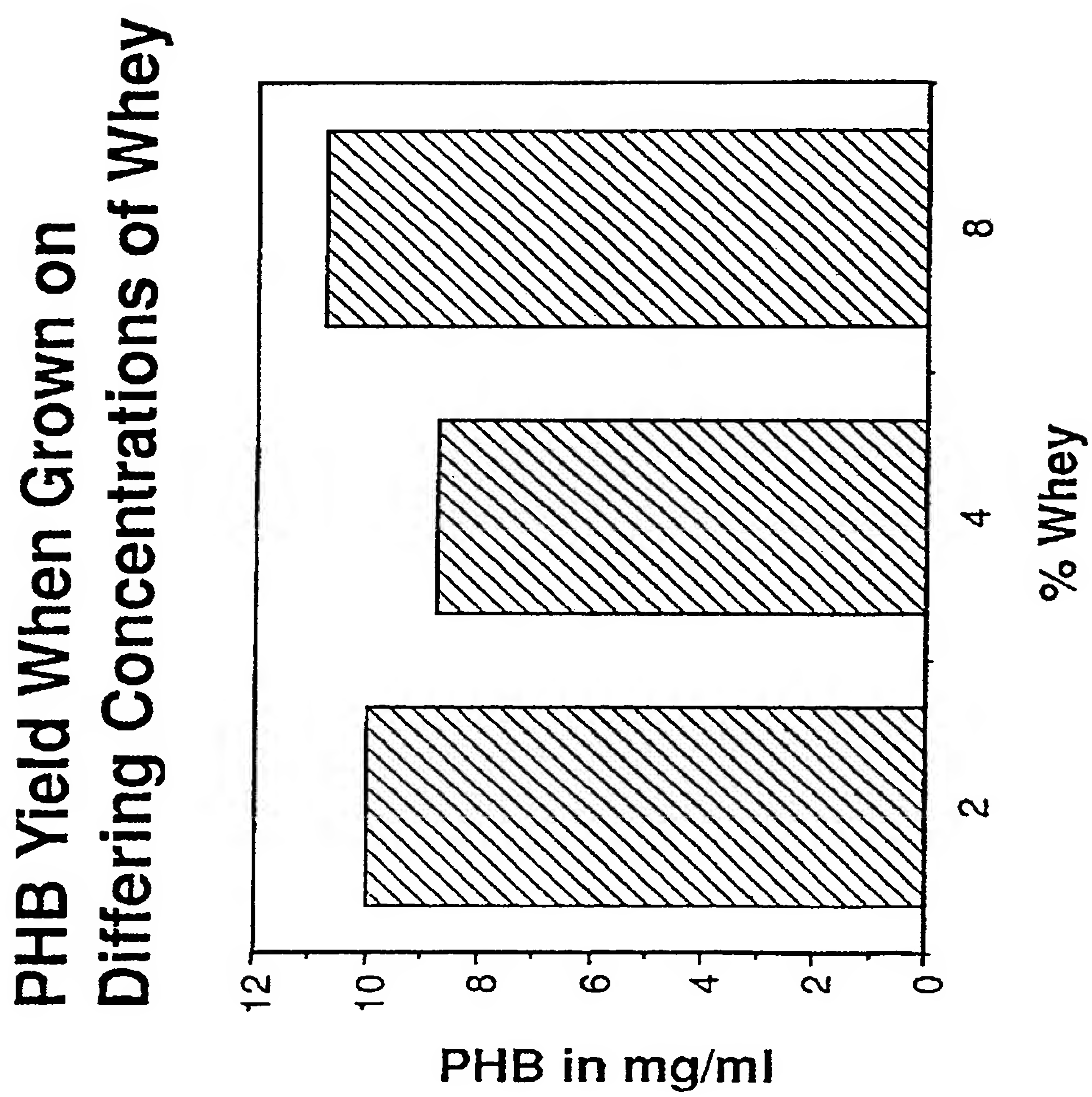
PHB Yield When Grown on
Differing Concentrations of Whey
PHB in mg/ml
0
2
4
6
8
10
12
% Whey
2
4
8

FIG. 2b

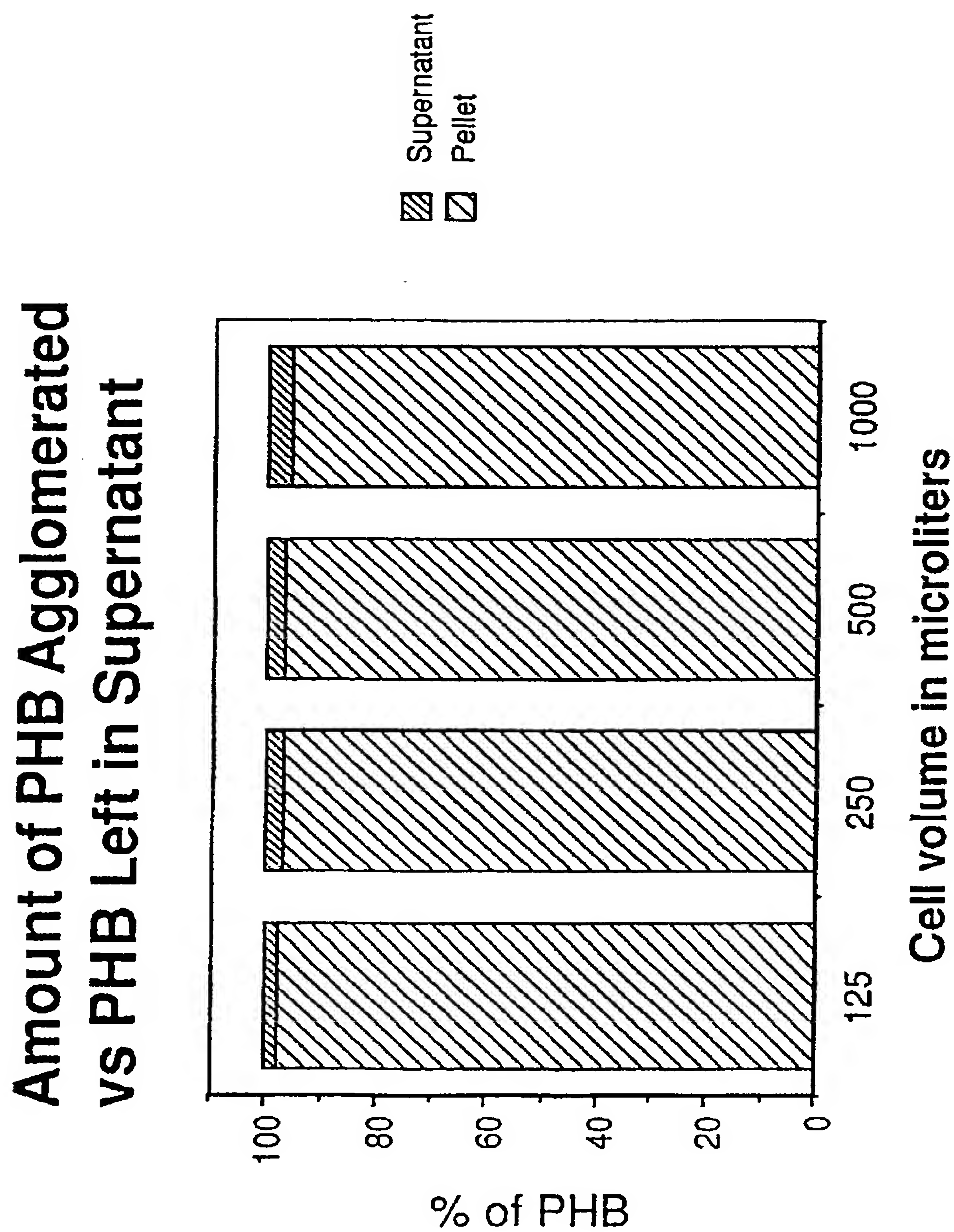
Amount of PHB Agglomerated
vs PHB Left in Supernatant
Supernatant
Pellet
% of PHB
100
80
60
40
20
0
125
250
500
1000
Cell volume in microliters

FIG. 3

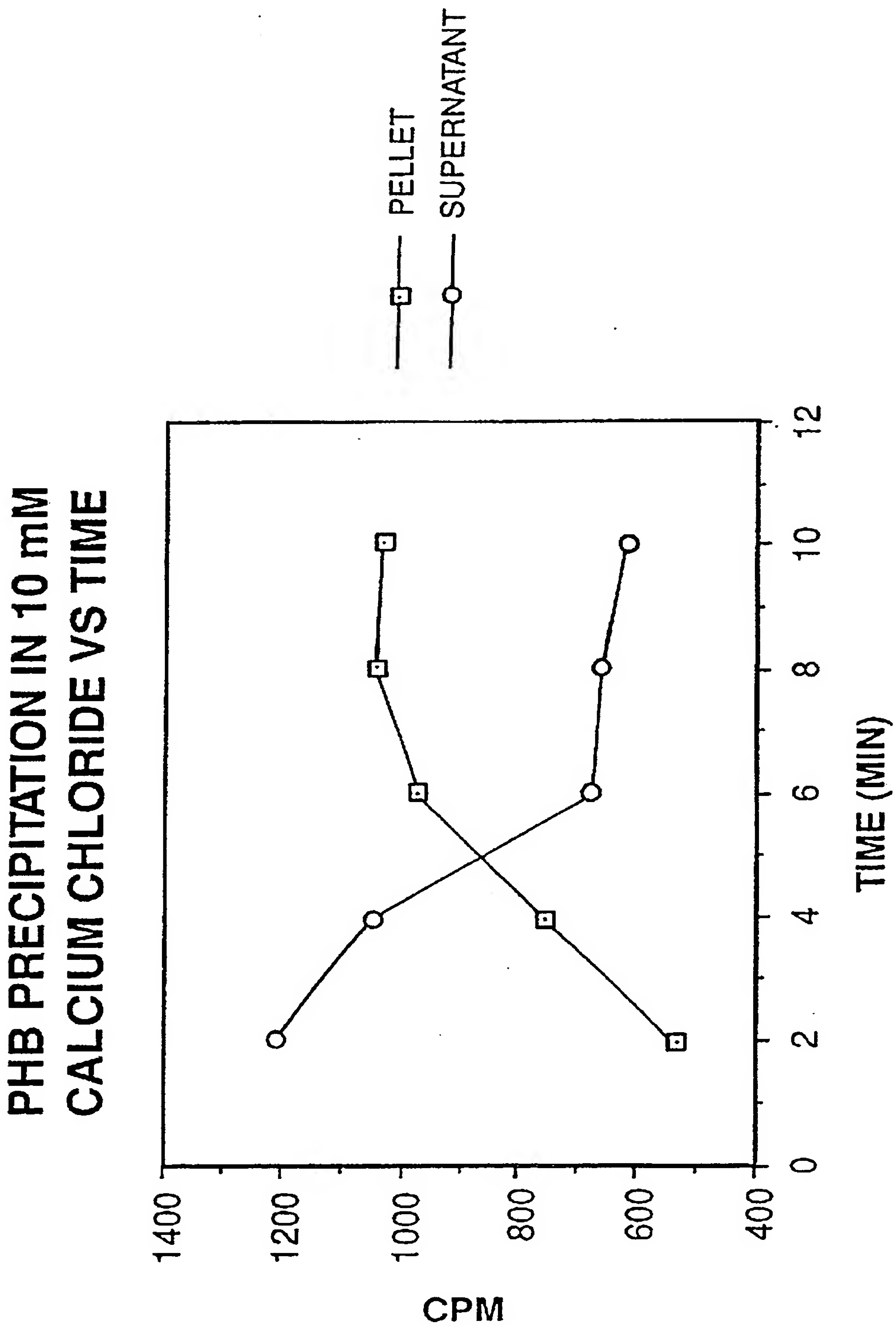
PHB PRECIPITATION IN 10 mM
CALCIUM CHLORIDE VS TIME
PELLET
SUPERNATANT
CPM
1400
1200
1000
800
600
400
TIME (MIN)
0
2
4
6
8
10
12

FIG. 4

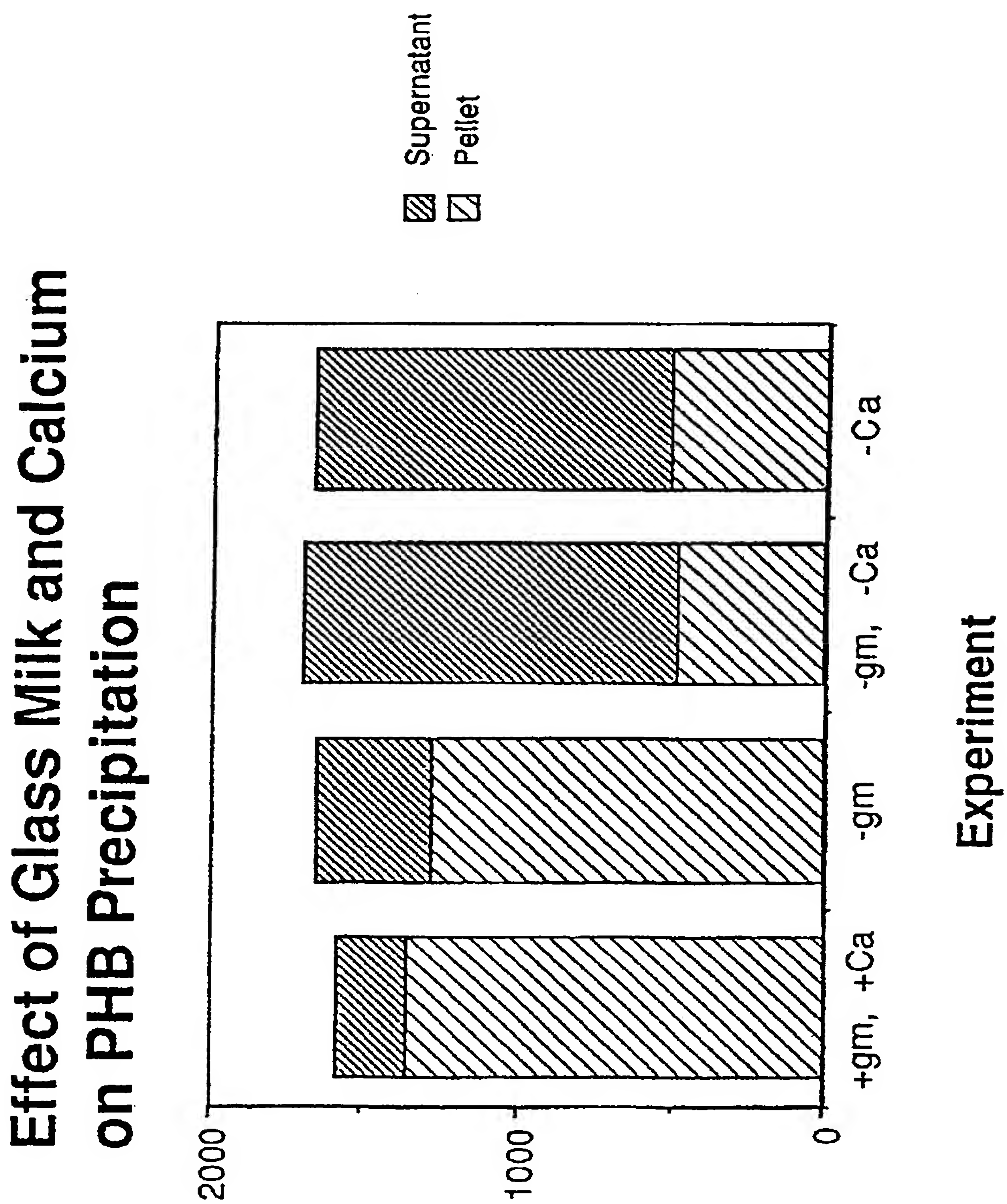
Effect of Glass Milk and Calcium on PHB Precipitation
Supernatant
Pellet
2000
1000
0
+gm, +Ca
-gm
-gm, -Ca
-Ca
Experiment

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03547

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P7/ 40, 7/02; C12N1/20, 15/00; C07H15/12, 1/06
U.S.Cl.: 435/136,155,243,252.3,252.33,280,320.1; 536/1.1,26,27,28,29,124,127

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	435/136, 155, 243, 252.3, 252.33, 280, 320.1; 536/1.1, 26, 27, 28, 29, 124, 127

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Online Search Words: poly hydrotybutyrate, transform? recombin?, clon?, bacteria, ferment?, whey, aglommerat?, magnesium, calcium, ion?

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	JOURNAL OF BACTERIOLOGY, Vol. 170, No. 10, issued October 1988, Slater et al., "Cloning and Expression in Escherichia coli of the Alcaligenes eutrophus H16 Poly-beta-Hydroxybutyrate Biosynthetic Pathway", pages 4431-4436, see entire document.	1,6,8 1-26
Y	TRENDS IN BIOTECHNOLOGY, Vol. 5, issued September, 1987, Byrom, "Polymer Synthesis by Microorganisms: Technology and Economics", pages 246-250, see entire document.	1-26
Y	US,A,4,743,453 (Ahern et al.) 10 May 1988, see Abstract.	10-13, 17
Y	Ayers et al., "Microbiology of foods" published 1980 by W.H. Freeman and Company (San Francisco), see pages 191-192.	10-13, 17
Y	US,A,4,396,763 (Tsuchiya et al.) 02 August 1983, see column 1, lines 29-31 and 43-47.	10-21
P,Y	US,A,4,950,749 (Johal et al.) 21 August 1990, see abstract.	14-16, 18-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 September 1991	17 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Stephanie Zitomer for

International Application No. PCT/US91/03547

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers .... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ________, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3.☐ Claim numbers ____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. claims 1-9,13-17,24,25 drawn to a first product (host) and first process of use (produce PHB).

II. Claims 10-12 drawn to a second product (culture medium).

See attached sheet.

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. telephone practice

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

CONTINUATION SECTION VI

III. Claims 18-21, 26 drawn to second process (recovering PHB).

IV. Claim 22 drawn to a third product (DNA sequence).

V. Claim 23 drawn to a fourth product (plasmid vector).